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## Effect of moderate walnut consumption on lipid profile, arterial stiffness, and platelet activation in humans

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### Abstract

**Background/Objectives**—A large intake of walnuts may improve lipid profile and endothelial function. The effect of moderate walnut consumption is not known. We investigated whether a moderate intake of walnuts would affect lipid profile, arterial stiffness and platelet activation in healthy volunteers.

**Subjects/Methods**—Thirty healthy males were recruited into a single-blind randomised controlled crossover trial of 4 weeks dietary walnut supplementation (15 g/day) and 4 weeks control (no walnuts). Arterial stiffness was assessed using pulse waveform analysis to determine the augmentation index and augmented pressure. Platelet activation was determined using flow cytometry to measure circulating platelet-monocyte aggregates.

**Results**—There were no differences in lipid profile after 4 weeks of walnut supplementation compared with control. Dietary intake of alpha-linolenic acid was increased during the walnut diet ( $2.1 \pm 0.4$  g/day versus  $0.7 \pm 0.4$  g/day,  $P < 0.0001$ ). There were no differences in augmentation index or augmented pressure during walnut supplementation. Walnut supplementation did not affect platelet-monocyte aggregation.

**Conclusions**—Dietary intervention with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in man. Our results suggest that the potentially beneficial cardiac effects of walnuts may not be apparent at lower and more practical levels of consumption.

### Keywords

walnuts; lipids; platelet activation; arterial stiffness; cardiovascular disease

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All authors contributed to the study design. JD, SA, AJ, FC, and KL were responsible for data collection and analysis. All authors contributed to data interpretation. JD, JS, DN and AF contributed to the writing of the manuscript.

#### CONFERENCE PRESENTATION

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#### Conflicts of interest statement

None of the authors have any conflicts of interest in relation to this work.

## Introduction

Frequent consumption of nuts may be protective against coronary heart disease (Hu and Stampfer, 1999). Nuts are rich in unsaturated fatty acids and contain potentially beneficial compounds including anti-oxidants, L-arginine, fibre, and folic acid (Kris-Etherton *et al.*, 1999). Walnuts are unique because they also contain high levels of  $\alpha$ -linolenic acid, a plant derived omega-3 fatty acid that may confer additional cardiac protection (de Lorgeril and Salen, 2004). Dietary intervention studies have demonstrated that a large intake of walnuts can reduce LDL cholesterol (Banel and Hu, 2009). In addition, walnuts may improve endothelial function in patients with hyperlipidemia or diabetes (Ma *et al.*, 2010; Ros *et al.*, 2004). However, the precise mechanisms through which walnuts confer their putative cardiac benefits remains uncertain.

The endothelium plays a critical role in the regulation of vascular smooth muscle tone, thrombosis and inflammation. Vascular smooth muscle tone is an important determinant of central arterial pressure and arterial stiffness. Large artery stiffness, wave reflections and central pulse pressure are inversely associated with endothelial function and can be measured non-invasively using pulse waveform analysis (McEniery *et al.*, 2006). The endothelium also regulates local platelet activation and aggregation through the release of paracrine factors, such as nitric oxide and prostacyclin. Platelet-leukocyte aggregates are highly sensitive markers of platelet activation that are closely related to impaired endothelial vasomotor function (Robinson *et al.*, 2006). They contribute to the development of atherothrombosis and are elevated in coronary heart disease and acute coronary syndromes (Sarma *et al.*, 2002).

Marine-derived long chain omega-3 fatty acids reduce arterial compliance and platelet activation (Din *et al.*, 2004; Din *et al.*, 2008). We therefore hypothesised that dietary intervention with walnuts, rich in plant-derived omega-3 fatty acids, would reduce arterial stiffness and platelet activation in humans. Previous intervention studies have asked participants to consume large amounts of walnuts (30-108 grams or approximately 8-27 whole walnuts daily) providing up to 50% of the total dietary fat intake. This proportion of fat energy from walnuts may be too high to be considered practical, and no previous trials have assessed the effect of a more realistic intake of walnuts. In the present study we examined the effect of a moderate intake of walnuts on lipid profile, arterial stiffness and platelet activation.

## Subjects and Methods

### Study participants and design

Thirty healthy male volunteers were enrolled into the study. Exclusion criteria included those taking regular medication, those with clinical evidence of atherosclerotic vascular disease, hypertension, diabetes mellitus, hypercholesterolemia, an intercurrent illness likely to be associated with an acute phase inflammatory response, and renal or hepatic insufficiency. Ethical approval was obtained from the Lothian Research Ethics Committee and all subjects provided written informed consent.

Participants were randomised to 4 weeks of walnut supplementation (15 g / day) or 4 weeks of control (no walnuts) in a single-blind randomised crossover trial. Block randomization was used and investigators were blinded as to whether participants were in the walnut intervention or control period. The walnut intake was calculated to provide levels of alpha-linolenic acid similar to the current American Heart Association recommendations and trials showing a reduction in cardiac events with diets rich in alpha-linolenic acid (de Lorgeril *et*

*al.*, 1994; Kris-Etherton *et al.*, 2002). Lipid profile, platelet-leukocyte aggregation, and arterial stiffness were assessed at the end of each 4 week period.

### Dietary intervention

Participants were provided with walnuts at the beginning of the dietary intervention phase and asked to consume 15 g of walnuts daily in addition to their habitual diets. No other specific dietary advice was provided and there were no restrictions on calorie or fat intake. For the control phase of the study, participants were simply asked to continue with their usual diets. Similar to previous studies, we did not include a washout period between diets as diet-induced lipoprotein changes stabilize in less than 4 weeks (Kris-Etherton and Dietschy, 1997). Participants completed a 3-day weighed food diary to assess intake of omega-3 fatty acids during each phase of the study. Subsequent dietary analysis was performed by a qualified dietician using the CompEat Nutritional Analysis Software program (Nutrition Systems, UK), enabled with McCance and Widdowson's reference food composition tables

### Blood collection protocol

Peripheral venous blood was drawn from a large antecubital vein with a 19 gauge needle and anticoagulated with the direct thrombin inhibitor D-Phenylalanine-L-prolyl-L-arginine chloromethyl ketone (75  $\mu$ mol, Cambridge Biosciences). Whole blood was then immunolabelled within 5 minutes of phlebotomy for subsequent flow cytometric analysis of platelet-monocyte aggregates.

### Flow cytometry

The following reagents were used: fluorescein isothiocyanate-conjugated CD42a (GRP-P, IgG1), and isotype control IgG1 were obtained from Serotec Ltd (Oxford, UK), phycoerythrin-conjugated CD14 (Tuk-4, IgG2a) was obtained from Dako Cytomation (Buckinghamshire, UK) and FACS-Lyse was obtained from Becton-Dickinson (Cowley, UK). The methods are as previously described (Harding *et al.*, 2007). Briefly, aliquots of whole blood were incubated with anti-CD14, anti-CD42a and isotype matched controls. Thereafter, samples were fixed and the red cells lysed. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm wavelength laser (Beckman Coulter, High Wycombe, UK). Samples were initially analysed with the flow cytometer triggered on forward scatter and then by triggering on FL-2 to identify CD14 positive monocytes. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. All results are expressed as percentage of positive cells. Analyses were performed using EXPO 32 software (Beckman Coulter, High Wycombe, UK). The mean coefficient of variation for the percentage of platelet-monocyte aggregates is 7.8%.

### Pulse waveform analysis

Arterial stiffness was measured non-invasively with the SphygmoCor system (AtCor Medical). Measurements were taken in a quiet, temperature controlled room after subjects had been in the recumbent position for a 15 minute rest period. Peripheral pressure waveforms were obtained using applanation tonometry of the radial artery with a pressure sensitive micromanometer (Millar Instruments). A generalised transfer function was used to derive the corresponding aortic pressure waveform. The first systolic peak of this waveform is a result of left ventricular ejection and the second systolic peak is caused by wave reflection from the periphery. The augmented pressure was defined as the difference between the first and second systolic peaks. The augmentation index was the augmented pressure expressed as a percentage of the pulse pressure. Our reproducibility measures demonstrate within-observer differences of  $0.4 \pm 1.5\%$  for the augmentation index and  $0.3 \pm$

0.6 mm Hg for augmented pressure. Peripheral blood pressure measurements were performed using an automated upper arm blood pressure monitor (Omron 705IT, Omron Healthcare).

### Statistical methods

Continuous variables are reported as mean  $\pm$  standard deviation. Statistical analyses were performed using 2-tailed Student's *t*-tests. All calculations were performed using GraphPad Prism (Graph Pad Software). Statistical significance was taken  $p < 0.05$ .

## Results

### Baseline characteristics

Baseline characteristics for the study population are given in Table 1. Study participants were young and had a normal body mass index, blood pressure and lipid profile. Two subjects were cigarette smokers and the average alcohol intake was within recommended limits ( $15 \pm 10$  units per week). In terms of ethnic origin, 22 participants were North European Caucasians, 5 were Indian Asians and 3 were of Far East Asian origin.

### Effect of walnut supplementation on lipid profile and intake of omega 3 fatty acids

Total and LDL cholesterol were lower than baseline after both the walnut intervention and control diets ( $P < 0.01$ ), but there were no differences in lipid profile between groups at the end of each 4 week period (Table 2). Dietary intake of alpha-linolenic acid was markedly increased during the walnut diet ( $2.1 \pm 0.4$  g/day *versus*  $0.7 \pm 0.4$  g/day;  $P < 0.0001$ ). There were no changes in consumption of the marine-derived omega-3 fatty acids eicosapentaenoic acid ( $0.2 \pm 0.2$  g/day *versus*  $0.2 \pm 0.3$  g/day) or docosahexaenoic acid ( $0.2 \pm 0.3$  g/day *versus*  $0.3 \pm 0.3$  g/day) during walnut supplementation compared with control.

### Effect of walnut supplementation on measures of arterial stiffness

Dietary intervention with walnuts did not affect heart rate, peripheral blood pressure, or central aortic pressures compared with control (Table 3). Measures of arterial stiffness did not change from baseline after either walnut supplementation or control, and there were no differences observed in augmentation index ( $-6.6 \pm 6.5$  % *versus*  $-8.4 \pm 6.3$  %), or augmented pressure ( $-2.2 \pm 2.1$  mmHg *versus*  $-2.7 \pm 2.4$  mmHg) or at the end of each 4 week period (Figure 1).

### Effect of walnut supplementation on platelet-leukocyte aggregation

Platelet-monocyte aggregates were lower compared with baseline after both the walnut intervention ( $18.5 \pm 7.2$  % *versus*  $22.0 \pm 6.8$ %,  $P = 0.004$ ) and control diets ( $19.5 \pm 7.2$  % *versus*  $22.0 \pm 6.8$ %;  $P = 0.048$ ). Platelet-neutrophil aggregates also fell from baseline after the walnut intervention ( $5.2 \pm 1.8$  % *versus*  $7.2 \pm 3.0$ %,  $P = 0.0003$ ) and control diets ( $5.5 \pm 2.2$  % *versus*  $7.2 \pm 3.0$ %;  $P = 0.0027$ ). However, there were no differences in platelet-monocyte aggregation ( $18.5 \pm 7.2$  % *versus*  $19.5 \pm 7.2$  %) or platelet-neutrophil aggregation ( $5.2 \pm 1.8$  % *versus*  $5.5 \pm 2.2$ %) between walnut intervention and control diets at the end of each 4 week period (Figure 2).

## Discussion

We have demonstrated that dietary supplementation with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in healthy subjects. This is in contrast with previous studies which have shown that heavy consumption of walnuts as part of a low-fat or "Mediterranean" diet can reduce LDL cholesterol and improve endothelial

function. The present study is the first dietary intervention trial to assess the efficacy of moderate rather than large walnut consumption on markers of cardiovascular risk.

Data on the effects of walnut consumption on lipid profile have been inconsistent. Whilst several randomised trials have found a reduction in total or LDL cholesterol with walnuts (Iwamoto *et al.*, 2002; Ros *et al.*, 2004; Sabate *et al.*, 1993; Tapsell *et al.*, 2004; Torabian *et al.*, 2010; Zambon *et al.*, 2000), others have shown no effect (Chisholm *et al.*, 1998; Morgan *et al.*, 2002; Mukuddem-Petersen *et al.*, 2007; Spaccarotella *et al.*, 2008; Tapsell *et al.*, 2009). Overall, a meta-analysis of 11 trials found that high-walnut enriched diets reduced total and LDL cholesterol by 4.9% and 6.7%, respectively (Banel and Hu, 2009).

The most likely explanation for the lack of effect on lipid parameters in our study is the lower amount of walnuts consumed (15 grams daily) compared to previous trials. Studies demonstrating reductions (6-16%) in serum LDL cholesterol concentrations with walnuts have required participants to consume between 40 to 84 grams of walnuts per day (Iwamoto *et al.*, 2002; Ros *et al.*, 2004; Sabate *et al.*, 1993; Zambon *et al.*, 2000). Furthermore, in these studies walnuts isocalorically replaced other fat containing foods as part of low fat, low cholesterol or “Mediterranean” style diets, whilst our study participants were free-living. It is not possible to know whether the beneficial lipid effects in previous studies were specifically due to walnut intake or the replacement of other sources of dietary fat.

Most previous trials have investigated patients with hyperlipidaemia, and the relatively low baseline cholesterol in our study population may have contributed to the neutral lipid results. A recent trial found that lipid-lowering with walnut supplementation was only evident in patients with higher cholesterol levels; there was no benefit in those with baseline cholesterol concentrations <4.94 mmol/l (Torabian *et al.*, 2010). The only two trials in normolipidaemic patients where walnuts reduced cholesterol levels required a large intake of >50 grams per day (Iwamoto *et al.*, 2002; Sabate *et al.*, 1993). It is possible that supplementation with a smaller amount of walnuts is not sufficient to alter lipid parameters in a young, low-risk, normolipidaemic population.

The present study is the first trial to assess the effect of walnut consumption on arterial stiffness and platelet activation. We have previously demonstrated that dietary intervention with fish rich in marine-derived omega 3 fatty acids reduces platelet-monocyte aggregates, a highly sensitive marker of platelet activation (Din *et al.*, 2008). However, supplementation with walnuts rich in plant-derived omega 3 fatty acids did not reduce platelet-monocyte aggregates, and there was also no change in the augmentation index or augmented pressure. Although we did not study and cannot exclude an effect with heavy walnut intake, our results indicate that any potential cardiac benefits of moderate walnut consumption are unlikely to be mediated through effects on platelet activation or arterial stiffness.

The large amount of walnuts consumed in many of the previous dietary intervention trials provided up to 20% of total energy and 55% of daily total fat intake. There have been concerns that this proportion of fat energy from walnuts may be too high to be practical or sustainable in a non-research setting (Banel and Hu, 2009; Feldman, 2002). In 2004, the Food and Drug Administration issued a qualified health claim for walnuts in response to a petition from the California Walnut Commission (Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2004). Their report recognised that the clinical studies used high daily walnut intakes and that there were no data from which to extrapolate beneficial effects to lower amounts. The present study provides some data to address these concerns, and suggests that a smaller and more practical intake of walnuts may not be sufficient to improve lipid parameters or cardiovascular risk markers.



Our study has potential limitations which should be recognised. Firstly, the sample size is relatively small. However, the power of the study is augmented by the cross over design and was considered sufficient to detect important changes in outcome measures. Previous studies demonstrating improved lipid parameters and endothelial function with walnuts have had fewer participants. We therefore believe it is unlikely that our neutral results are due to a lack of power. Secondly, we cannot fully exclude a lack of adherence to the assigned diet as a cause of our neutral findings. Although the weighed food diary analysis suggested complete adherence, this is a self-reported and imperfect measure of compliance.

Finally, we did not include a run-in period prior to randomisation. All eligible subjects were randomised and an intention-to-treat analysis performed in order to maximise the applicability and external validity of the study. Whilst there were no differences between the diets at the end of each 4 week period, both the walnut intervention and control diets reduced cholesterol concentrations and platelet-leukocyte aggregates compared with baseline. These changes may reflect a modification in participants' dietary habits after randomisation, independent of their dietary assignment, and could have reduced our ability to detect subtle differences between diets.

## Conclusions

We have demonstrated that dietary intervention with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in healthy male volunteers. This is in contrast to previous studies in which large amounts of walnuts, as part of iso-caloric low fat diets, were found to reduce serum LDL cholesterol concentrations and improve endothelial function. Our results suggest that the potentially beneficial effects of walnuts on lipid parameters and cardiovascular biomarkers may not be apparent at lower and more practical levels of consumption. However, we cannot exclude the possibility that the neutral results are due to either small study size or lack of adherence to the walnut intervention. Further studies are necessary to determine whether larger amounts of walnuts might affect arterial stiffness or platelet activation. Longer trials are required to establish if the higher levels of walnut consumption associated with improved lipid profiles or endothelial function can be sustained over time and reduce clinical end-points.

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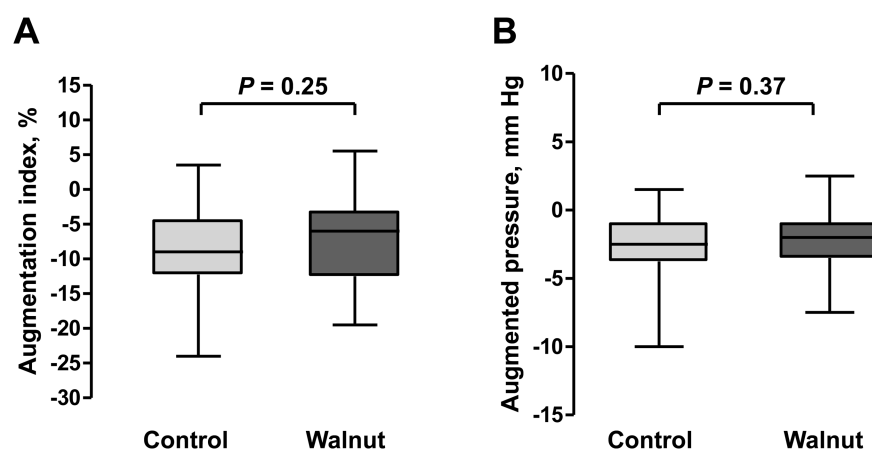
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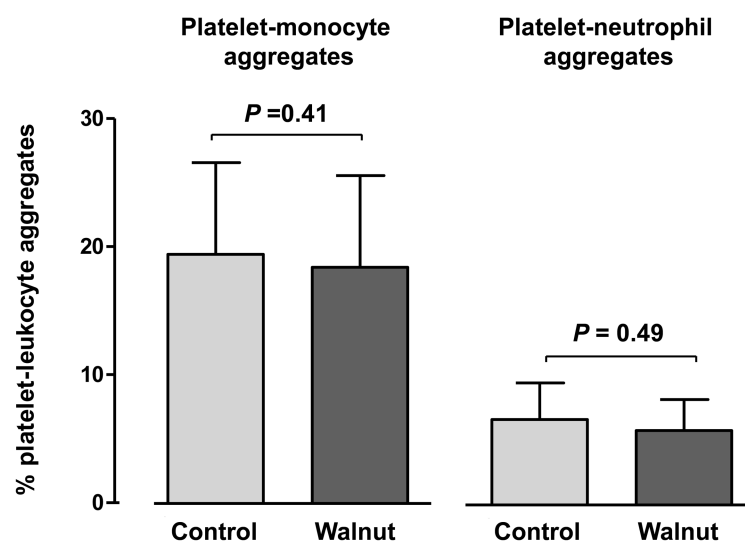
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**Figure 1.** Effect of dietary intervention with walnuts on augmentation index (A) and augmented pressure (B). Median (horizontal line), interquartile range (box) and 95% confidence intervals (bars).



**Figure 2.** Effect of dietary intervention with walnuts on platelet-monocyte and platelet-leukocyte aggregates. Mean $\pm$ SD.

**TABLE 1****Baseline characteristics**

Age, years	23±3
Body mass index, kg/m <sup>2</sup>	24.5±2.3
Systolic blood pressure, mm Hg	117±9
Diastolic blood pressure, mm Hg	60±7
Total cholesterol, mmol/l	4.57±1.06
LDL cholesterol, mmol/l	2.70±0.92
HDL cholesterol, mmol/l	1.32±0.24
Triacylglycerol, mmol/l	1.22±0.58
Augmentation index, %	-5.3±6.9
Augmented pressure, mm Hg	-1.8±2.5
Platelet-monocyte aggregates, %	22.0±6.8
Platelet-neutrophil aggregates, %	7.2±3.0

Mean±SD

**TABLE 2**

Effect of walnut supplementation on serum lipid profile

	Control	Walnuts	<i>P</i> value
Total cholesterol, mmol/l	4.37±1.01	4.25±0.99	0.11
<i>change from baseline, mmol/l</i>	<i>-0.33±0.39</i>	<i>-0.20±0.37</i>	
LDL cholesterol, mmol/l	2.46±0.85	2.30±0.84	0.10
<i>change from baseline, mmol/l</i>	<i>-0.40±0.47</i>	<i>-0.24±0.37</i>	
HDL cholesterol, mmol/l	1.24±0.19	1.24±0.26	0.85
<i>change from baseline, mmol/l</i>	<i>-0.09±0.19</i>	<i>-0.08±0.15</i>	
Chol:HDL chol ratio	3.47±0.85	3.39±0.85	0.63
<i>change from baseline, mmol/l</i>	<i>-0.13±0.61</i>	<i>-0.05±0.62</i>	
Triacylglycerol, mmol/l	1.45±0.74	1.55±0.93	0.61
<i>change from baseline, mmol/l</i>	<i>0.33±0.86</i>	<i>0.23±0.59</i>	

Mean±SD.

**Table 3****Haemodynamic effects of walnut supplementation**

	<b>Control</b>	<b>Walnuts</b>	<b>P Value</b>
Heart rate, beats per minute	64±8	68±11	0.08
Peripheral systolic blood pressure, mmHg	117±8	120±10	0.07
Peripheral diastolic blood pressure, mmHg	62±5	63±8	0.66
Peripheral pulse pressure, mmHg	54±8	57±9	0.11
Mean arterial pressure, mmHg	77±6	79±8	0.29
Central aortic systolic pressure, mmHg	96±7	98±8	0.09
Central aortic diastolic pressure, mmHg	63±5	64±8	0.54

Mean±SD.